Genistein Affects *HER2* Protein Concentration, Activation and Promoter Regulation via Estrogen Receptor-and non-Estrogen Receptor-Mediated Mechanisms

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Abstract

The *HER2* proto-oncogene, a member of the epidermal growth factor receptor family, is over expressed in 20-30% of breast cancers. Genistein, the main soy isoflavone, interacts with estrogen receptors and it is also a potent tyrosine kinase inhibitor. Previously, our lab found that genistein delayed mammary tumor onset in transgenic mice that over express *HER2* gene. Our goal was to define the mechanism through which genistein affects mammary tumorigenesis in *HER2* over-expressing mice. We hypothesized that genistein inhibits *HER2* activation and expression by both ER-dependent and ER-independent mechanisms. Genistein inhibited total *HER2* protein expression and tyrosine phosphorylation in BT-474, an ERα (-) and ERβ (+) human breast cancer cell line, however, E2 had no effect. Taken together, these data suggest that genistein has an ER-independent inhibitory effect, presumably, through tyrosine kinase inhibition activity.

Although E2+ER and over-expression of *HER2* can promote mammary tumorigenesis, an inverse relationship between ER expression and *HER2* over-expression has been found in human breast cancer. We cloned a 500bp promoter region upstream of the transcription initiation site. At low doses, genistein mimicked E2 and down-regulated *HER2* promoter reporter in BT-474 cells when co-transfected with ERα but not with ERβ. However, at high doses, genistein inhibition was independent on ERα. In conclusion, genistein acts through ERα at low doses and independently from ERα at high doses, and through a combination of these two pathways, genistein regulates *HER2* protein

expression, tyrosine phosphorylation and *HER2* promoter activity. These data support genistein's chemo-prevention and potential chemo-therapeutic roles in breast cancer.

INTRODUCTION

Breast cancer is the most common cancer and is the second leading cause of cancer death among women in the United States. There are two important receptors whose expression are used as predictive factors in breast cancer; estrogen receptor alpha (ERα) and *HER2* receptor (*ErbB2, neu*) [1]. The American Society of Clinical Oncology has recommended the assessment of *HER2* in all breast tumors, either at the time of diagnosis or recurrence [2].

HER2 is the second member of the Human Epidermal Growth Factor Receptor Family (EGFR) which is composed of 4 members; *HER1*, *HER2*, *HER3* and *HER4*. The *HER2* receptor gene maps to chromosome 17q21 and is a 1255 amino acid, 185kd trans-membrane glycoprotein [3]. All four HER receptors are composed of an extra-cellular ligand binding domain, transmembrane lipophillic domain, and an intracellular domain with tyrosine kinase catalytic activity [3]. Although *HER2* does not have known ligands, *HER2* protein has an important role in normal cell growth and differentiation. However, over-expression of *HER2* is linked to the development of many human cancers [4].

HER2 receptor is over-expressed in 20-30% of primary breast cancer cases, and its presence can impede the anti-proliferative effect of hormonal

therapy [5]. Moreover, a high level of *HER2* expression is associated with lymph node positive, high nuclear grade, negative hormone receptor status, and high proliferative activity [4]. Because *HER2* has an important role in tumorigenesis, it has been investigated as a target for cancer therapy. One of these approaches is the use of tyrosine kinase inhibitors [6]. It has been found that blockade of EGFR function with the EGFR specific tyrosine kinase inhibitor ZD1839 (Iressa) inhibits phosphorylation of the *HER2* receptor and growth of *HER2* over-expressing breast carcinoma cells. Moreover, a combined molecular approach using a combination of ZD1839 and the *HER2* antibody, herceptin, had more anti-tumor effectiveness [7] than either approach alone. Although both *HER2* and ligand activated ERα are growth promoting [8], an inverse relationship was found between over-expression of *HER2* and expression of ERα in human breast cancer specimens [9]. Natural alternatives, like phytoestrogens, that target tumorigenesis at multiple molecular sites such as *HER2* and ER may be effective in breast cancer prevention and/or treatment.

The phytoestrogen genistein, the main isoflavone in soy, has been proposed to be the agent responsible for lowering the rate of breast cancer in Asian women [10]. Genistein has been proposed to work through many mechanisms [11,12]. It can interact with both estrogen receptors (ERα and ERβ), with preference for ERβ, demonstrating both agonist and antagonist effects [13,14]. Genistein is also a potent inhibitor of tyrosine kinase [15], and

may reduce *in vivo* tyrosine kinase activity, specifically epidermal growth factor (EGF) receptor phosphorylation in rat prostate [16].

Several lines of evidence indicate that genistein is protective in breast cancer. First, epidemiological studies have shown that the age-adjusted death rates from breast cancer are 2 to 8 fold lower in Asia compared to Western countries, suggesting that dietary factors play an important role in reducing breast cancer risk in Asian countries [17]. In addition, immigration studies showed that first generation Asian immigrants have low rates of breast cancer compared to the second and the subsequent generations [18]. Secondly, the average daily intake of soy in Asian culture is about 50g per day compared to 1g per day in Western culture [18]. Thirdly, many cell culture and experimental models provide evidence for genistein's role in preventing breast cancer [reviewed in [19]]. Finally, in previous work, we observed a significant delay in mammary tumor development in genistein-treated transgenic mice expressing the activated *HER2/neu* oncogene under the control of mouse mammary tumor virus (MMTV) promoter [20].

The overall goal of our present work is to define the mechanism by which genistein affects *HER2* activation and gene expression. Our working hypothesis is that genistein transcriptionally regulates *HER2* expression through estrogen-dependent and estrogen-independent mechanisms. In this manuscript, we found low doses of genistein repress *HER2* protein and

promoter activation via an ERα-dependent mechanism. This observation provides an explanation for the chemopreventive role of genistein where ERα is present in normal breast epithelium. Furthermore, we also showed that high doses of genistein repressed *HER2* protein and promoter activation in an ER-independent mechanism. The ER-independent inhibition of *HER2* transcription using high genistein doses could be a potential therapy for *HER2* over-expressing breast cancer patients who usually lack ER expression.

Materials and Methods

Chemicals

Genistein was obtained from LC Laboratories (Woborn, MA), dissolved in DMSO and stored as 100 mM stocks at -20^{o}C. 17β estradiol and β-tubulin antibody (cat #T7816) were obtained from Sigma Chemical Co. (St. Louis, MO). The human reactive polyclonal antibodies for ERα (PA1-308) and ERβ (PA1-312) were purchased from Affinity Bio Reagents (Golden, CO). The human reactive rabbit polyclonal for HER-2 antibodies (cat # sc-284-R) and the human reactive rabbit polyclonal for phosphorylated Tyr 1248 *HER2* (cat # sc-12352-R) were purchased from Santa Cruz Biotechnology, INC.

Plasmids

Human *HER2* promoter construct was prepared by cloning a 525bp region (-495/+30) in relation to the transcription initiation site [21]. XhoI and Hind III restriction sites were inserted by using the following primers: forward primer sequence: 5' ttccagaagatactcgaggggggtcctgga 3' and reverse primer sequence:

5’gctgcccgggggaagcttctggtttctccg 3’. Amplified DNA products were ligated into the basic PGL-3 luciferase vector (Promega, Madison, WI). After transformation, sequences from independent colonies were verified at the DNA core facility of the University of Missouri, Columbia.

ERα and ERβ expression vectors cloned in pcDNA Zeo 3.1(+) expression vector were prepared as described previously [22].

Cell culture and transfection experiments

BT-474 human breast cancer cells from ATCC were maintained in Hybri-Care Medium (Modified Dulbecco's medium) supplemented with 10% fetal bovine serum. For transient transfection experiments, BT-474 cells were plated in 24-well plates in phenol red free medium and charcoal stripped serum for 24 hours and transiently transfected using Fugene 6 (Roche). Cells were transfected with 5 ng ERα or ERβ plasmids and 400 ng *HER2* promoter reporter vector. After 24 hours, transfected cells were treated with the assigned treatment for 20 hours. Transfection experiments were normalized to co-transfected *pRL-SV40* renilla vector (Promega, Madison, WI). Luciferase assays were done using the Dual Luciferase Assay kit (Promega).

Western Blotting

BT-474 cells were washed with cold PBS then lysed in ice-cold buffer (50 mM Tris-HCL, 150 mM NaCl, I mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 100 mM PMSF (pH 7.4) with freshly

added protease inhibitor cocktail on ice for 30 minutes. The cells were scraped, collected in microfuge tubes and sonicated for 30 seconds. The cell lysate was cleared by centrifugation at 14000 x g for 15 minutes at 4^{o}C and the supernatant (total cell lysate) was used or immediately stored at –80^{o}C. The total cellular protein concentration was determined by the DC Bio-Rad assay (Bio Rad laboratories, Hercules, CA). For Western blotting, 10µg protein was resolved over 8-12% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were transversely cut according to the molecular weight marker to use the upper part to show protein expression for the target protein and the lower part was used for analysis of internal control expression (β-actin or β-tubulin). Membranes were incubated in blocking buffer (5% nonfat dry milk/1%Tween 20; in 20mM TBS, pH 7.6) for two hours at room temperature, then with appropriate polyclonal primary antibody overnight at 4^{o}C, followed by incubation with secondary antibody horseradish peroxidase conjugate (Amersham Life Science Inc., Arlington Height, IL). Bands were detected by chemiluminescence and autoradiography using XAR-5 film (Eastman Kodak CO., Rochester, NY).

Results

Characterization of BT-474 human breast cancer cell line.

Previously, we have shown that genistein represses cancer development in MMTV/*neu* mice that over-express *HER2* [20]. To further understand the mechanism of these results, we studied *in vitro* the effect of genistein on *HER2* expression and activation. BT-474 human breast cancer cells have been chosen

as our model because they over-expresses *HER2* protein [23], which was confirmed by western blot (figure 1A). We also characterized BT-474 for ER expression [24]. Although BT-474 cells have been reported as ERα (+) cells [25], others found weak ERα expression [26], a third group reported BT-474 to be an ER negative cell line [27]. Our characterization confirmed that the BT-474 cells we used had minimal or negative ERα expression compared to the well known ERα (+), *HER2*(+) ZR75.1 cells and ER(+), *HER2* (-) MCF-7 cells (figure 1B). Characterization of ERβ expression revealed that BT-474 cells express similar amount of ERβ protein as MCF-7 cells (figure 1C).

Genistein, but not 17βestradiol, inhibited *HER2* protein expression and phosphorylation.

Genistein has been shown to be effective treatment in inhibiting ER (-) breast cancer cell growth [28,29] which is of clinical importance because most of *HER2* over-expressing breast tumors are ER(-) and resistant to hormonal treatment. We found genistein inhibited BT-474 cell growth while E2 did not (data not shown).

The ability of genistein to regulate proliferation of the over-expressing *HER2*, BT-474 cells suggests that genistein could regulate *HER2* expression and/or activation. The effect of genistein on *HER2* protein activation and phosphorylation was determined in BT-474. Treatment of BT-474 cells for 5 days with 0, 1 μM, 10 μM and 25 μM genistein resulted in a significant dose dependent inhibition of *HER2* protein expression (figure 2A, 2B) and

phosphorylation (figure 3A, 3B). However, treatment with 0, 0.01 nM, 0.1 nM, 1.0 nM, or 10 nM 17β-estradiol for 5 days did not affect *HER2* protein expression (figure 2C, 2D) or phosphorylation (figure 3C, 3D).

Because genistein, but not E2 treatment, repressed *HER2* protein expression or activation in this ERα negative cell line, we conclude that genistein's inhibitory action is likely through an ER-independent pathway, presumably through its ability to act as tyrosine kinase inhibitor.

Role of ERα in the effect of genistein dose response on *HER2* promoter luciferase construct.

To determine if genistein can regulate *HER2* expression transcriptionally, we cloned the minimum active *HER2* promoter (-495/+30) [21] into the luciferase reporter vector PGL-3 basic. This reporter assay system was used to determine the effect of genistein on *HER2* transcriptional regulation in the absence or presence of co-expressed ERα. BT-474 cells were transfected with the *HER2* promoter luciferase construct with either ERα expression vector or an empty vector. Treatments were added for 20 hours.

At low doses, genistein (0.1 μM, 1.0 μM) mimicked E2 and significantly down-regulated *HER2* promoter reporter in BT-474 cells co-transfected with ERα expression vector (figure 4). However, at high doses (10μM, 50 μM, 75μM, 100 μM), genistein generated similar repression on HER2 promoter reporter in both the presence and absence of ERα. In the absence of ERα, a dose dependent

inhibition of *HER2* promoter activity by genistein was observed. The *HER2* promoter activity was inhibited by 60% at 50μM concentration. Similar results were obtained using COS-1 cells (data not shown). To our knowledge, this is the first evidence that a botanical compound inhibits *HER2* promoter activation and reveals the role of ERα in its action.

To confirm the role of ERα in mediating the inhibitory effect of genistein on *HER2* protein activation level, expression of phosphorylated HER2 was determined by western blot. Genistein at 1.0 μM mimicked E2 inhibition of *HER2* activation when BT-474 cells were co-transfected with ERα (figure 5A and 5B).

Role of ERβ and genistein on *HER2* promoter

To define the role of ERβ in *HER2* promoter and protein activation, BT-474 cells were transfected with *HER2* promoter luciferase construct with ERβ expression vector (5, 10 or 20 ng). Assigned treatments (control, 10 nM E2, or 1.0 μM genistein) were added for 20 hours. ERβ had no effect on *HER2* promotor activation as determined by luciferase reporter assays (figure 6A) and no effect on *HER2* protein activation as determined by western blot (figures 6B and 6C).

Discussion

Genistein has been suggested to have a protective role in breast cancer *in vivo* as well as *in vitro* models [19]. One oncogene that has been shown to be inhibited by genistein is the *HER2* protein. In our study, we have shown that 25 μM genistein for 5 days inhibited total *HER2* protein expression in ERα (-) BT-474 cells by 60% (figure 2A, 2B). Consistent with our findings, Li et al, 1999 [28] showed that 30μM genistein treatment for 3 days inhibited *HER2* protein expression in MDA-MB-435 ER(-) human breast cancer cells by 50%. They focused on the effect of genistein on cell apoptosis in the absence of ERα. In addition, the aim of that study was to determine the effect of genistein on *HER2* protein dynamics as they used *HER2* expression vectors. We took a different research approach, providing evidence for transcriptional inhibition of human *HER2* at the promoter level by genistein and showing the protective role of ERα but not ERβ in the BT-474 human breast cancer cell line.

Genistein is a specific inhibitor of tyrosine protein kinase [15] and thereby, could inhibit the tyrosine kinase domain in *HER2* protein. We found that genistein starting at 1.0 μM concentration significantly inhibited *HER2* phosphorylation after 5 days of treatment (figure 3A, 3B), however, E2 did not change *HER2* phosphorylation in ERα (-) BT-474 breast cancer cells (figure 3C, 3D). Among studies that have been done to show the tyrosine kinase inhibitory effect of genistein, Katdare, et al., 2002 [30] showed by immuno-histochemistry that 10μM genistein inhibited *HER2* protein and tyrosine phosphorylation in 184-

B5/HER cells. The focus of their research was to show the effect of genistein on cell cycle and cell growth. Although they found that genistein did not inhibit EGFR phosphorylation, Dalu et al, 1998 [16] reported that genistein inhibited EGFR phosphorylation in the rat dorsolateral prostate. In addition, Akiyama et al. [15] reported significant inhibition of EGF phosphorylation by 1μg/ml genistein (equivalent to 3.7μM). In addition, genistein has been shown to alter protein tyrosine phosphorylation in human peripheral blood mononuclear cells after an oral dose of dietary amounts of genistein [31,32]. These ER-independent findings suggest that genistein could be used as drug therapy at high dose to target *HER2* over-expressing tumors that do not express ERα.

At the transcriptional level, AP-2 [33] and Ets binding sites [21] located in the *HER2* promoter are reported to play an important role in regulation of *HER2* gene expression. Although E2+ER and over-expression of *HER2* can promote mammary tumorigenesis, an inverse relationship between ER expression and *HER2* over-expression has been found in human breast cancer [9]. E2 has been found to down-regulate *HER2* gene expression through the AP-2 site in ERα (+) breast cancer cells [34]. We proposed that at low doses, genistein mimics E2 and down-regulates the *HER2* promoter through an ERα-dependent mechanism. This model could explain the preventive role of genistein on normal mammary gland that already has ERα. On the other hand, several Ets transcription factors are nuclear targets of signaling pathways including the MAP kinase pathway which is a downstream target of *HER2* activation. MAP kinase

phosphorylates Ets proteins at a particular serine or threonine residues [35]. We demonstrated that genistein presumably as a tyrosine kinase inhibitor, inhibited *HER2* phosphorylation (figure 3A and 3B). Therefore, we propose that genistein at doses high enough to inhibit the tyrosine kinase could inhibit *HER2* promoter in the absence of ERα. Because most of *HER2* over-expressing tumors are ERα-negative, this is likely a good model for treatment.

To determine if genistein can regulate HER2 expression transcriptionally, we cloned the minimum active human *HER2* promoter (-495/+30) into the luciferase reporter vector PGL-3 basic [21]. We demonstrated that at low doses, genistein mimicked E2 and significantly down-regulated *HER2* promoter reporter in BT-474 cells when co-transfected with ERα expression vector. However, at higher doses, there was no significant difference between with and without ERα co-transfection. Surprisingly, our transcriptional study showed ligand-independent inhibition of the human *HER2* promoter when ERα expression vector was co-transfected in BT-474 cells. Both the ligand-independent stimulation as well as inhibition has been reported. The ligand-independent stimulation of ERE reporter by wild type ERα has been shown [36]. On the other hand, ligand-independent ERα inhibition of cell migration and metastasis has been shown in MDA-MB-231 breast cancer cells suggesting a protective role of the unliganded ERα [37]. Interestingly, *HER2* over-expression has been co-related to cell migration and metastasis [28] suggesting the role of ligand-independent inhibitory role of ERα on *HER2* gene expression. In addition, ERα

ligand-independent inhibition has also been shown on the ERE luciferase reporter in MCF-7 cells when co-transfected with steroid co-repressor MRF-1 [38]. To locate the E2 inhibitory site in *HER2* promoter, an E2-dependent inhibition of a 218bp fragment of human *HER2* promoter has been shown previously in ZR75.1 and SKBR.3 cells but not using the whole promoter [34].

At the *HER2* protein activation level, after 20 hours treatment, genistein at 1.0 μM mimicked E2 and inhibited *HER2* phosphorylation when BT-474 was co-transfected with ERα. This supports speculation about the role of ERα in inhibiting *HER2* total protein expression as demonstrated by Russell and Hung [27] who proposed that ER might be a negative regulatory factor for *HER2* gene expression. Using western blotting, they found that total *HER2* protein expression was inhibited in MCF-7 and BT-474 co-transfected with ERα expression vector when treated with 10nM E2 for 4 days.

The biological interactions between ERs and *HER2* are complex. Although activation of *HER2* down-regulated ER expression in short-term and gave rise to an ER (-) phenotype in the long-term [25], it also promotes E2-independent transcription activity [39]. Conversely, E2 down-regulated *HER2* in human breast cancer cells [34]. Bai and Giguere [5] have shown differential effects of *HER2* over-expression on ERα and ERβ. They showed that expression of *HER2* leads ERα, but not ERβ, to recruit SRCs (steroid receptor

co-activators). The differential effect of *HER2* on ERα versus ERβ could be involved in turn, in the different effects of the two ERs on *HER2* expression.

In conclusion, our data support our hypothesis that genistein works through ERα at low doses and as a tyrosine kinase inhibitor at high doses to regulate *HER2*. Through a combination of these two pathways, genistein regulates *HER2* protein expression, tyrosine phosphorylation and *HER2* promoter activity. This finding could explain the protective role of genistein against breast cancer in general and specifically the highly metastatic form that over-expresses *HER2*. The demonstration of non ER-dependent inhibition of *HER2* at higher doses, raise the possibility of using this inexpensive natural compound, genistein, as single or adjuvant therapy in ERα (-) and *HER2* (+) breast cancer.

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Figure legends

Figure 1

Characterization of the BT-474 human breast cancer cell line by western blot analysis of *HER2* (1 A), ERα (1 B) and ERβ (1 C) protein expression. Cell extracts were resolved by gel electrophoresis, electro-blotted onto nitrocellulose membrane, and immuno-detected. Figure 1A, extracts of MCF-7 and BT-474 cells. Figure 1B; lane 1, ZR75-1 cell extract as *HER2* (+), ERα (+) positive control; lane 2, BT-474 cells extract; lane 3, BT-474 tansfected with 10ng ERα expression vector; lane 4, BT-474 cells transfected with 20ng ERα expression; lane 5, MCF-7 cells as positive control. Figure 1C, extracts of BT-474 cells and MCF-7 cells as positive control.

Figure 2

Effect of 17β-estradiol and genistein on *HER2* protein expression in BT-474 cells. Cells were plated in E2 free medium, treated the next day with A) 0, 1, 10, 25 μM genistein (2A) or with 0, 0.01, 0.1, 1.0, 10 nM E2 (2C) for 5 days. The meduim with treatment was replaced every 3 days. Figures 2B, and 2D, individual band from figures 2A, 2C respectively, were quantitated, and the ratio of *HER2* protein to the internal control protein in 3 independent experiments is expressed as mean ± SEM. Bars with * are significantly different from the control, $p < 0.05$.

Figure 3

Effect of 17β-estradiol and genistein on *HER2* protein activation in BT-474 cells. Cells were plated in E2 depleted medium, treated the next day with 0, 1, 10 or 25

μM genistein (3A) or with 0, 0.01, 0.1, 1.0 or 10 nM E2 (3C) for 5 days. The media with treatment were replaced every 3 days. In figures 3B, and 3D, individual band from figures 3A, 3C respectively, were quantitated, and a ratio of *HER2* phosphorylated protein to the internal control protein in 3 independent experiments depicted graphically as mean ± SEM. Bars with ***** are significantly different from the control, $p < 0.05$.

Figure 4

Effect of genistein dose response on *HER2* promoter/ luciferase construct in presence and absence of ERα in BT-474 cells. BT-474 cells were transiently transfected with 400ng HER2 promoter luciferase construct and 5ng expression vector as indicated. Results are expressed as mean ± SEM from 3 independent experiments run in duplicate. Control/-ERα has been normalized to 1. Treatments in the same experiment are taken as ratio to its control. Data were analyzed by one-way ANOVA and pair comparison of means was done by multiple t-test comparison. Means were considered significantly different at $p < 0.05$. Bars with same letters are not significantly different.

Figure 5

The effect of ERα co-transfection on HER2 protein activation in BT-474 cells.

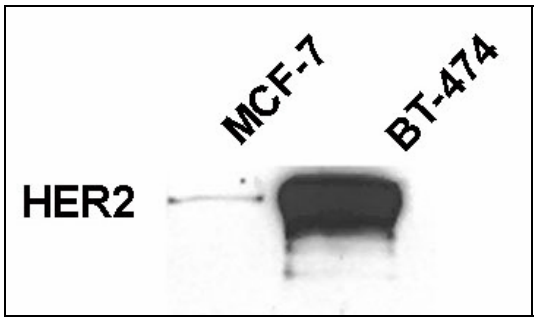
(A) Western blot analysis of phosphorylated HER2. Lysate from transfected BT-474 and treated with control (0), 10 nM 17β-estradiol (E2), 1.0 μM genistein (G) with (+) or without (-) 5 ng ERα expression vector co-transfection for 20 hours. (B) Individual bands were quantitated, and a ratio of *HER2* phosphorylated protein to the internal control protein in 3 independent experiments is expressed as mean ± SEM. Bar pairs with * are significantly different from its ERα-negative control using student t-test, $p < 0.05$.

Figure 6

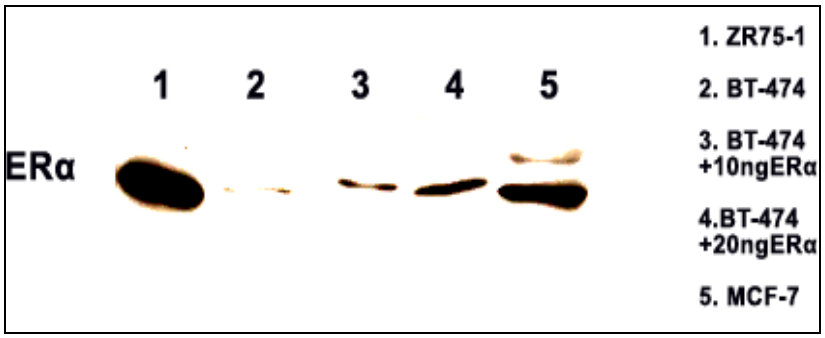
Role of ERβ co-transfection on *HER2* promoter and protein activation. A) BT-474 cells were transiently transfected with 400ng HER2 promoter luciferase construct and ERβ expression vector as indicated. Results are expressed as mean ± SEM from 3 independent experiments each with duplicate samples. B) Western blot analysis of phosphorylated HER2. Lysate from transfected BT-474 and treated with control (0), 10 nM 17β-estradiol (E2), or 1.0 μM genistein (G) with (+) or without (-) ERβ expression vector co-transfection for 20 hours. C) Individual band from figure 6B were quantitated, and a ratio of *HER2* phosphorylated protein to the internal control protein in two experiments done in duplicate depicted graphically as mean ± SEM.

Figure 1

1A



1B



1C

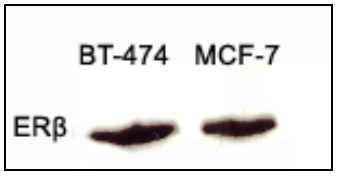


Figure 2

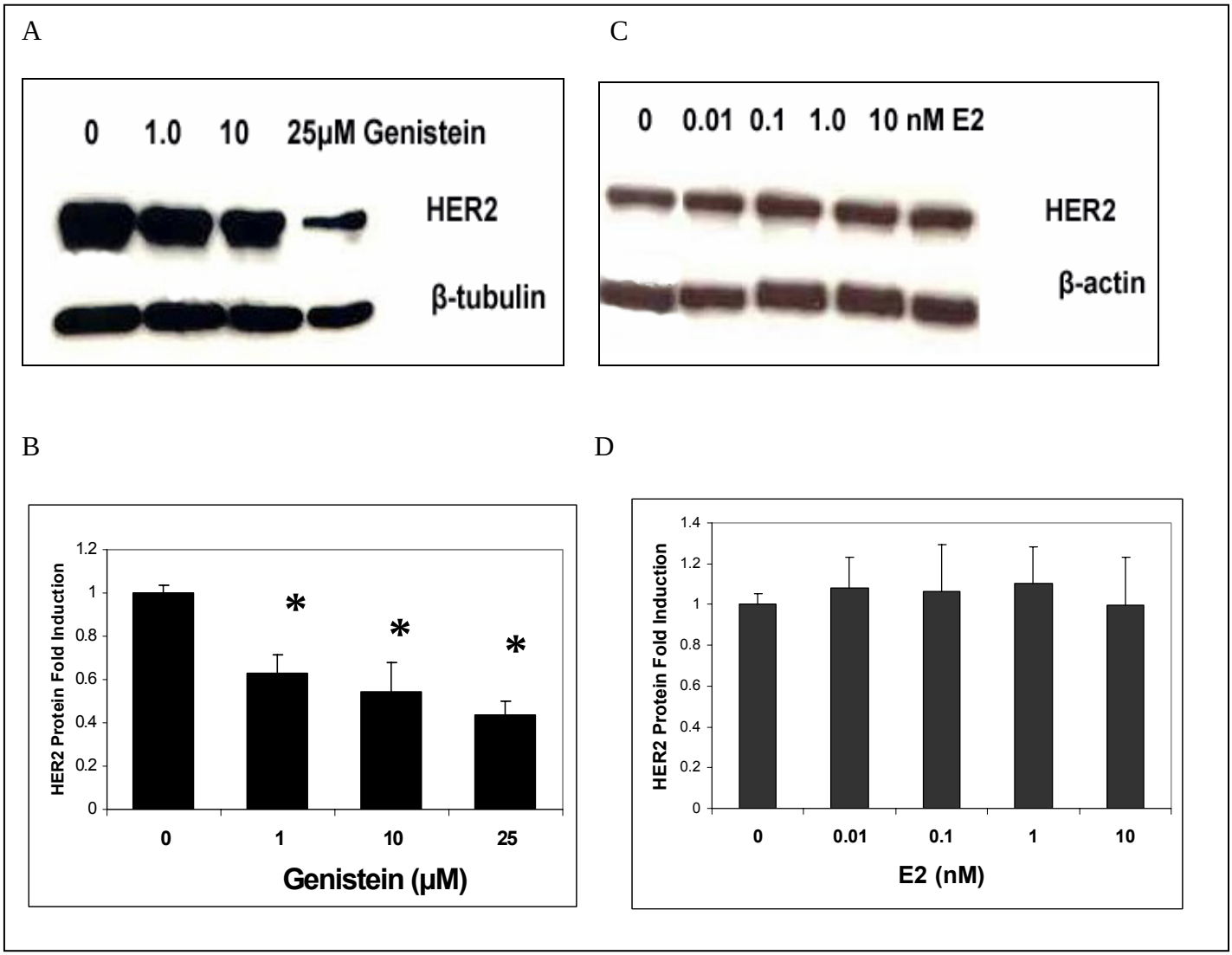

A
0 1.0 10 25μM Genistein
HER2
β-tubulin
C
0 0.01 0.1 1.0 10 nM E2
HER2
β-actin
B
HER2 Protein Fold Induction
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
0
1
10
25
Genistein (μM)
D
HER2 Protein Fold Induction
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.01
0.1
1
10
E2 (nM)

Figure 3

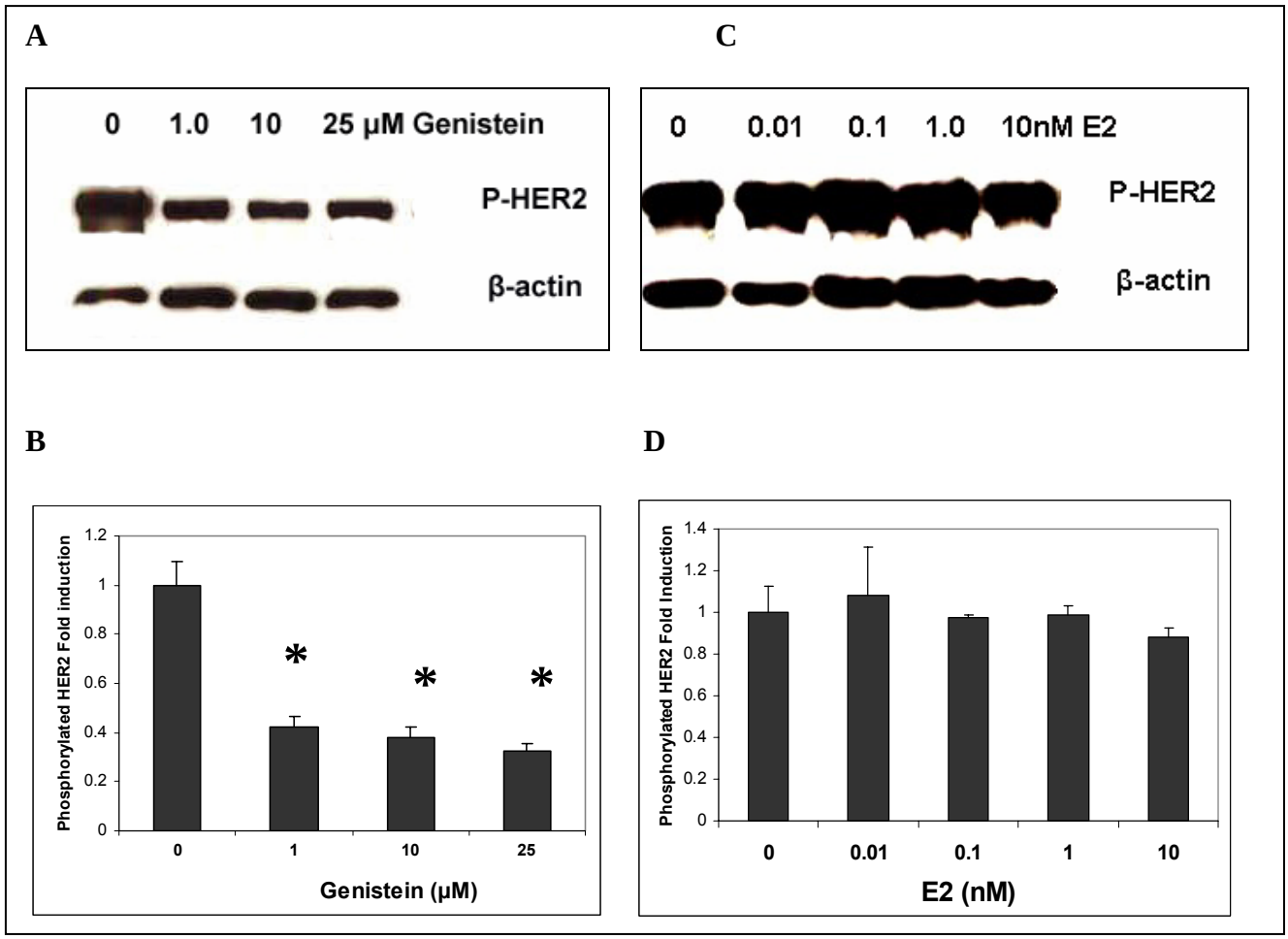

A
0 1.0 10 25 μM Genistein
P-HER2
β-actin
C
0 0.01 0.1 1.0 10nM E2
P-HER2
β-actin
B
Phosphorylated HER2 Fold induction
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
0
1
10
25
Genistein (μM)
D
Phosphorylated HER2 Fold Induction
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.01
0.1
1
10
E2 (nM)

Figure 4

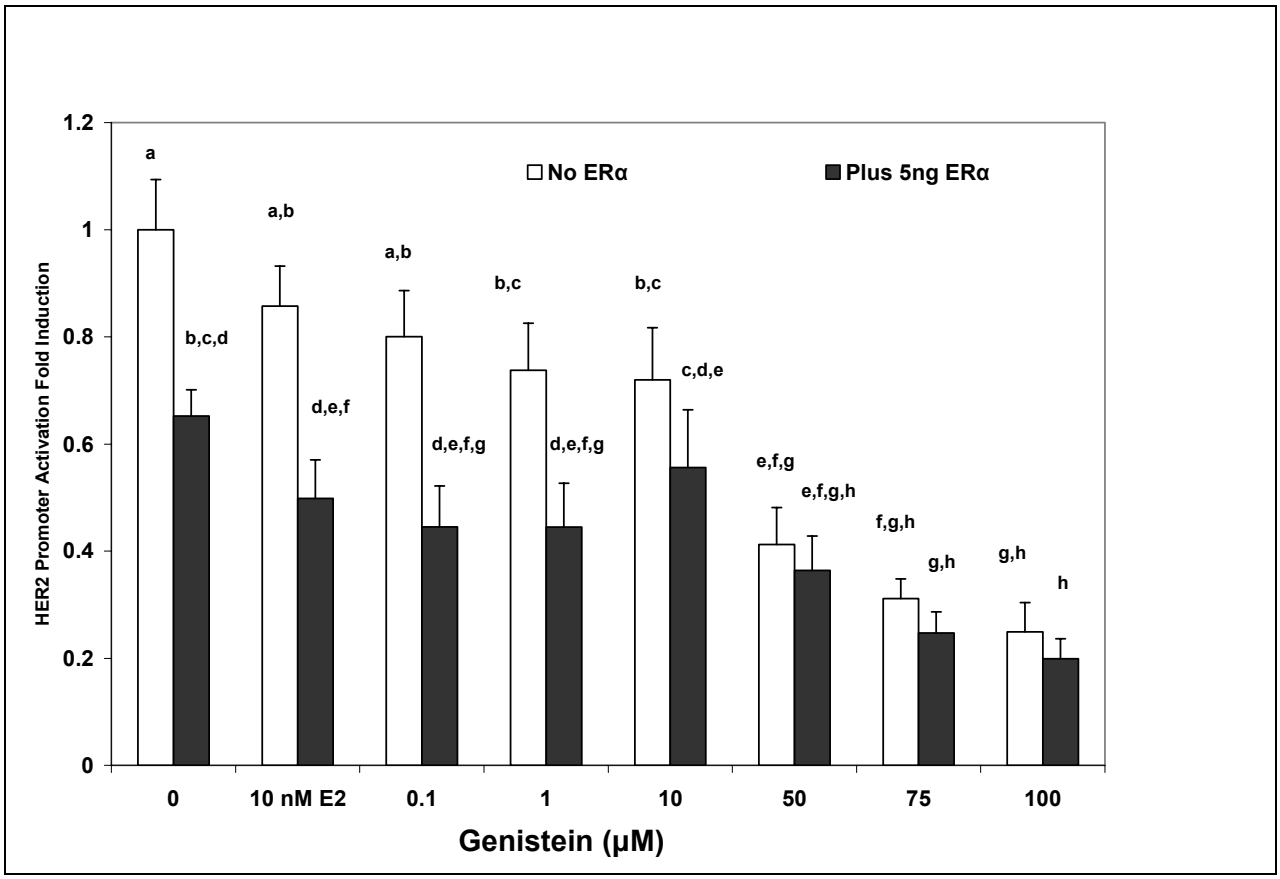
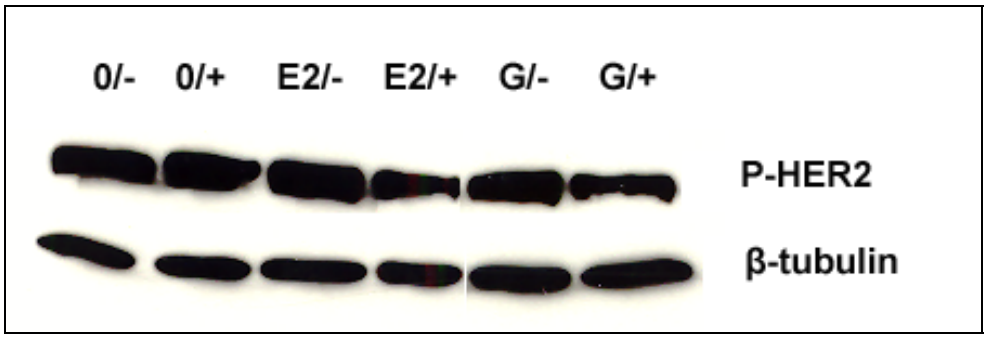

HER2 Promoter Activation Fold Induction
1.2
1
0.8
0.6
0.4
0.2
0
No ERα
Plus 5ng ERα
a
b,c,d
a,b
d,e,f
a,b
d,e,f,g
b,c
d,e,f,g
b,c
c,d,e
e,f,g
e,f,g,h
f,g,h
g,h
g,h
h
0
10 nM E2
0.1
1
10
50
75
100
Genistein (μM)

Figure 5

A


0/-
0/+
E2/-
E2/+
G/-
G/+
P-HER2
β-tubulin

B

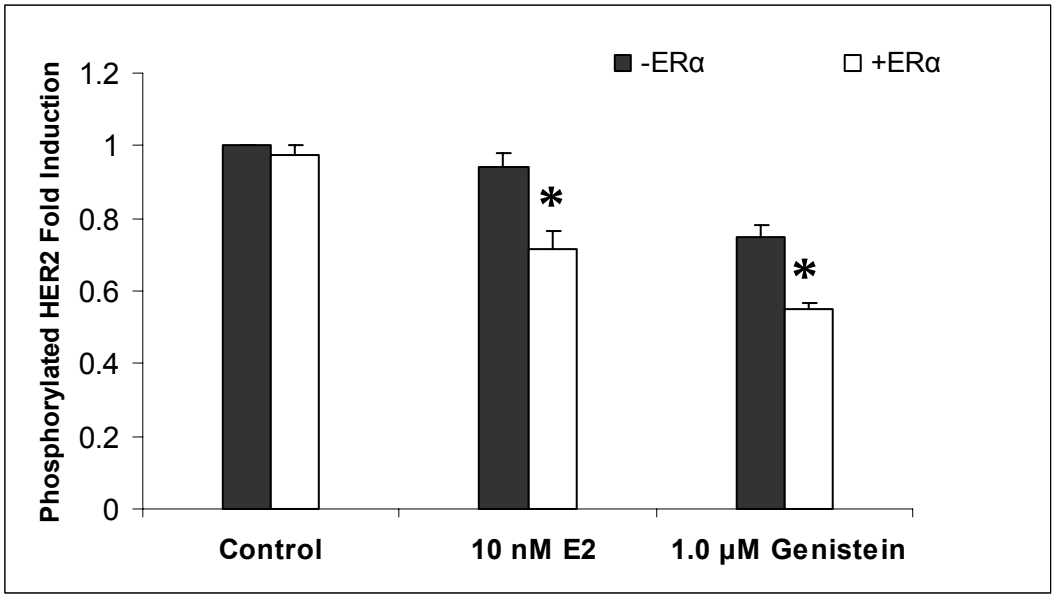
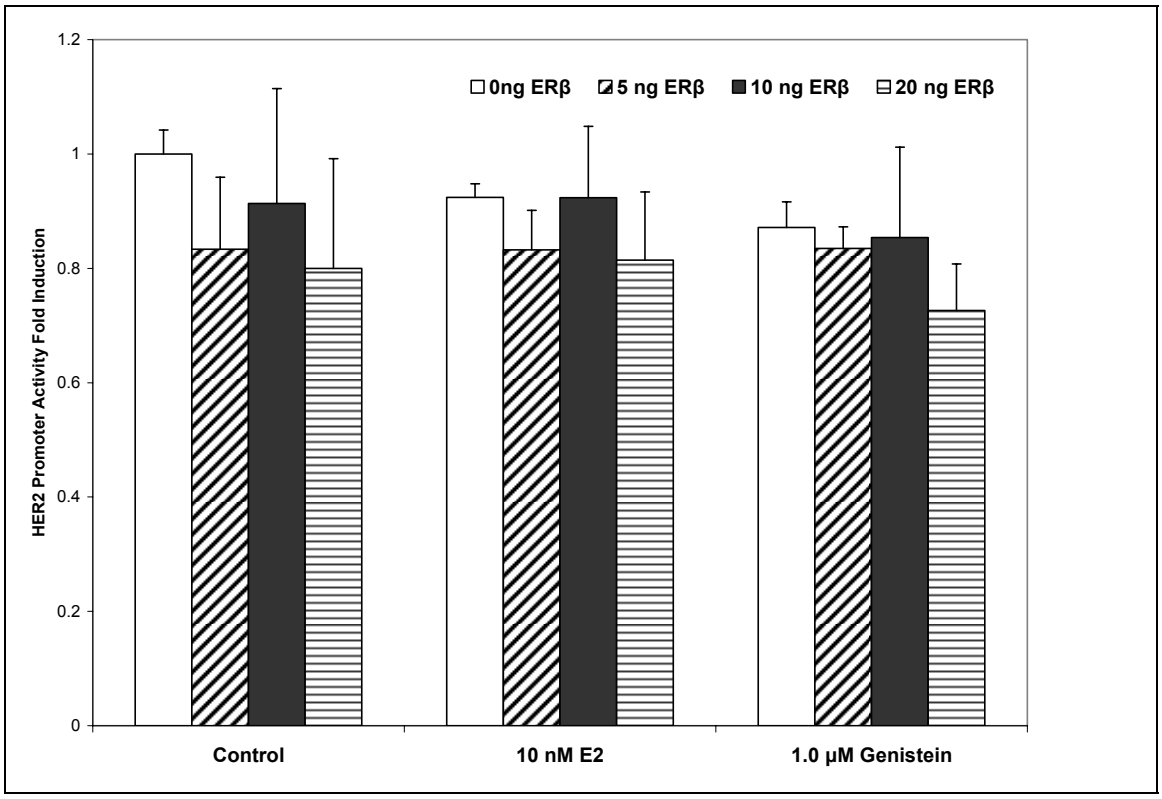

-ERα
+ERα
Phosphorylated HER2 Fold Induction
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Control
10 nM E2
1.0 µM Genistein

Figure 6

6A


1.2
1
0.8
0.6
0.4
0.2
0
HER2 Promoter Activity Fold Induction
0ng ERβ
5 ng ERβ
10 ng ERβ
20 ng ERβ
Control
10 nM E2
1.0 µM Genistein

6B

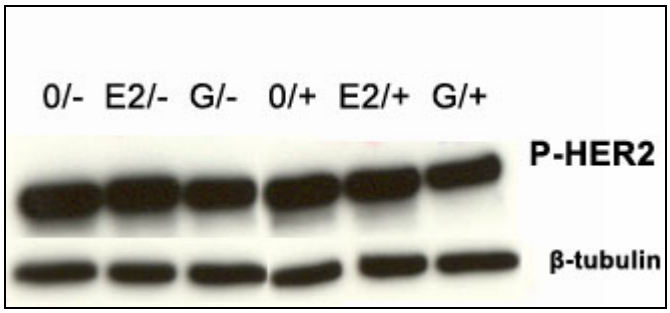
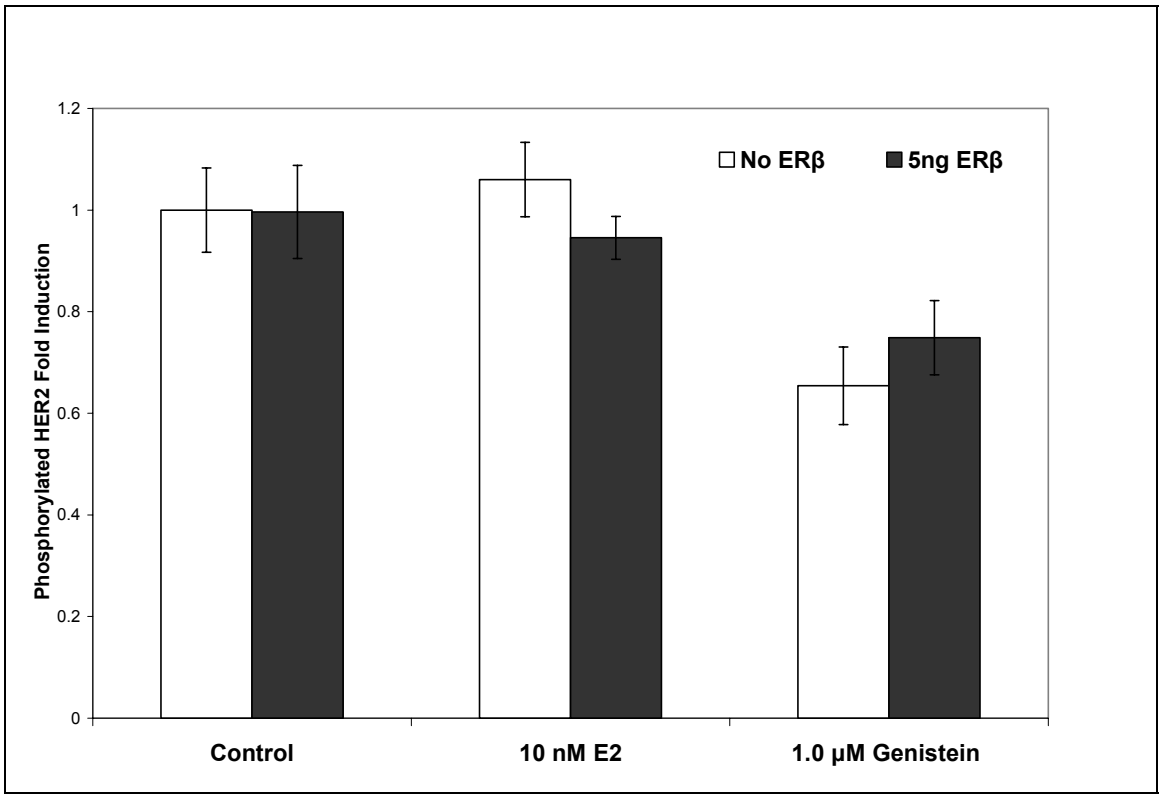

0/-
E2/-
G/-
0/+
E2/+
G/+
P-HER2
β-tubulin

Figure 6C


Phosphorylated HER2 Fold Induction
1.2
1
0.8
0.6
0.4
0.2
0
No ERβ
5ng ERβ
Control
10 nM E2
1.0 μM Genistein